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A Novel Quantitative Method for Analyzing the Distributions of Nanoparticles Between Different Tissue and Intracellular Compartments

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ABSTRACT

The penetration, translocation, and distribution of ultrafine and nanoparticles in tissues and cells are challenging issues in aerosol research. This article describes a set of novel quantitative microscopic methods for evaluating particle distributions within sectional images of tissues and cells by addressing the following questions: (1) is the observed distribution of particles between spatial compartments random? (2) Which compartments are preferentially targeted by particles? and (3) Does the observed particle distribution shift between different experimental groups? Each of these questions can be addressed by testing an appropriate null hypothesis. The methods all require observed particle distributions to be estimated by counting the number of particles associated with each defined compartment. For studying preferential labeling of compartments, the size of each of the compartments must also be estimated by counting the number of points of a randomly superimposed test grid that hit the different compartments. The latter provides information about the particle distribution that would be expected if the particles were randomly distributed, that is, the expected number of particles. From these data, we can calculate a relative deposition index (RDI) by dividing the observed number of particles by the expected number of particles. The RDI indicates whether the observed number of particles corresponds to that predicted solely by compartment size (for which RDI = 1). Within one group, the observed and expected particle distributions are compared by chi-squared analysis. The total chi-squared value indicates whether an observed distribution is random. If not, the partial chi-squared values help to identify those compartments that are preferential targets of the particles (RDI > 1). Particle distributions between different groups can be compared in a similar way by contingency table analysis. We first describe the preconditions and the way to implement these methods, then provide three worked examples, and finally discuss the advantages, pitfalls, and limitations of this method.

Key words: nanoparticles, transmission electron microscopy, intracellular distribution, stereology, chi-squared analysis, contingency table analysis

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INTRODUCTION

It is well known that ambient ultrafine particles (UFP)[1–4] and artificially produced nanoparticles (NP, <100 nm in diameter)[5–7] have the capacity to enter different cell types. Once translocated into the cells, they may cause several biological responses including the generation of reactive oxygen species,[8] the enhanced expression of proinflammatory cytokines,[9] and DNA strand breaks.[10] The intracellular occurrence of NP, either membrane-bound or membrane-free, is important with respect to the potential of NP to induce cellular responses. If NP can move freely (not membrane-bound) within a cell they may have direct access to organelles and the nucleus; this may greatly enhance their toxic potential. Therefore, the exact mechanisms of particle translocation and the intracellular trafficking of them are subjects of great interest for environmental and occupational medicine as well as pharmaceutical research. However, information about the intracellular localization of NP (which are assumed to behave in a similar fashion to UFP, and to simplify matters, will only be mentioned in the further text) often depends on the qualitative interpretation of "representative" images from electron microscopic thin sections or the reporting of absolute numbers of NP observed within different compartments. Although these reports are valuable because they have shown that NP differ from larger particles (>0.2 μm) by having unique properties of entering and traveling through cells,[6] a quantitative approach is needed to judge (1) whether the distribution of particles within an organ or cell type is random or preferential, (2) which compartments are preferential targets of particles, and (3) whether the observed distribution changes in different cell types, over time or, for example, due to variations in particle size, shape or surface, solubility, or chemistry. Combining molecular biology data with a quantitative analysis of the intracellular localization of NP will increase greatly our understanding of the interactions between different particles and organelles and help to identify the principal sites involved in the biological responses to NP exposure.

The best way to report particle distribution within a tissue or cell would be to determine the real number of particles in 3D and estimate the "concentration" of particles within a compartment. However, there is no way to do this directly and efficiently because the high resolution of the TEM is needed to identify particles at the nanoscale, and this requires the use of ultrathin sections that contain only a small part of the entire organ or cell. As a first approach to particle localization, one can count the number of particle profiles associated with different compartments from transmission electron microscope (TEM) sections but these data depend also on the sizes of compartments. For example, a larger organelle has a higher probability of being present in a section than a smaller one. Likewise, if two compartments contain the same "concentration" of particles, the number of particle profiles counted for the larger compartment will be higher than the one counted for the smaller compartment. This approach may be used for gaining a quick and preliminary insight into where particles reside but will not allow any further scientific conclusions. Instead, it is necessary to relate the number of particles to the sizes of cellular compartments (e.g., organelles), and this means that we need two sources of information: the number of particles associated with a compartment, and an estimation of the compartment size.

The number of particles can be counted directly from a set of randomly sampled micrographs. The size of the compartments can be obtained by applying design-based stereological methods as shown in Figure 1.[11] With these methods, we can exploit the fact that test grid points positioned randomly on sections will hit compartments in proportion to their sectional areas. Moreover, if the sections themselves are randomly located within a specimen, the test points will hit compartments in proportion to their volumes.[11,12]

Looked at from a broader perspective, the quantification of NP distribution is analogous to high-resolution immunocytochemistry where primary antibodies binding to a specific cellular antigen are visualized by secondary antibodies coupled with colloidal gold particles.[13] Under the TEM, only the gold particles—in representation of the antigen—are seen and attributed to the compartment on which they are observed to lie. In a series of papers, Mayhew and colleagues[14–17] have developed efficient methods for evaluating the cellular antigen distribution based on gold particle counts and null hypothesis comparisons of compartments within a cell or between cell types. The basic principles of quantifying particle numbers and compartment sizes

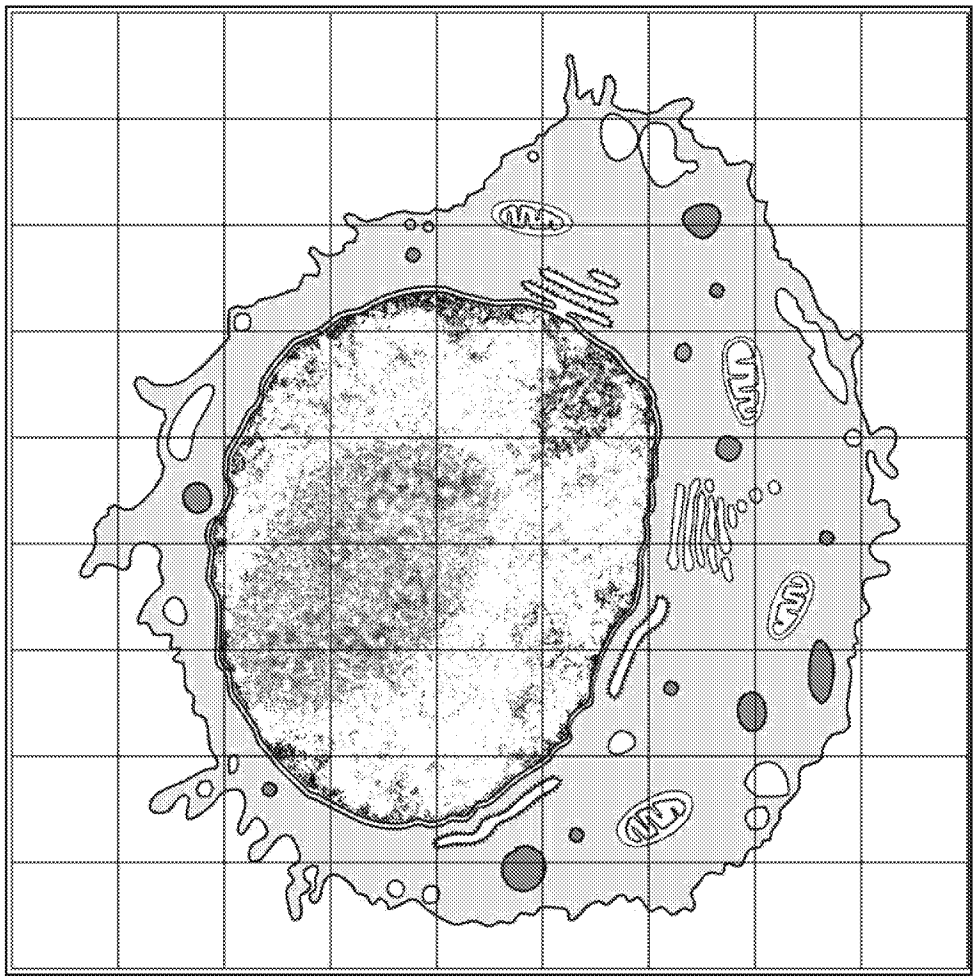

FIG. 1. The use of test points to determine the volume fraction of each defined compartment in relation to cell volume and, hence, the expected number of particles. A cell profile, taken from a larger set of images, all gathered by systematic uniform random sampling, is subjected to point counting. The intersections of the lattice are taken as points. The number of points hitting each defined compartment is counted (observed points). These numbers provide an estimate of the volume occupied by each compartment. If a number of particles were randomly distributed within the cell, the number of particles counted for each compartment would equal the observed number of points, that is, the observed points also represent an estimate for the expected number of particles.

and relating them to each other can be adopted for the purpose of analysing NP distributions. However, while those features which are unique to immunogold particles (e.g., antibody dilution, specificity, or signal-to-noise ratio) do not have to be considered in the current context, the analysis of NP distribution raises new and different problems that need careful consideration in the analysis, such as size or visibility of NP.

The present study illustrates how the quantitative methods successfully used in TEM immunocytochemistry,[16,18,19] can be applied to analyze NP distributions. In the following, we describe the exact ways of performing the analyses, and illustrate them by application to three different scenarios based on synthetic sets of data. In the discussion, we focus on the preconditions that have to be met for applying the methods, and summarize some potential advantages, pitfalls, and limitations that are unique to NP distribution analysis.

METHOD

We present three main scenarios which represent different possibilities in terms of the distributions of NP in two contexts. First, within-group studies in which NP distributions between different spatial compartments within a cell are being investigated. Second, between-group studies in which shifts in distributions between different

groups of cells are under investigation. We assume that sectional images of cells were generated by suitably randomized sampling procedures.[11,14,17] Thereafter, the method of dealing with each scenario in terms of counting, measuring, and testing a suitable null hypothesis is described.

Testing for randomness of distributions and preferential localization of particles

If NP could enter and leave subcellular compartments without restrictions, we could predict that they would be scattered randomly over all compartments. A corollary of such a distribution is that the number of particles in each compartment would be expected to be directly proportional to compartment volume. In that case, the "observed distribution" would equal the "expected distribution," that is, that predicted on the basis of randomness of location. Therefore, we can estimate the expected distribution from the fractional volumes of the cell occupied by compartments, and this can be achieved by randomly superposing a grid of test points over randomly located test fields and simply counting the number of points on each selected compartment (Fig. 1).

From the total number of observed particles, N_O(total), and the numbers of observed points, P, the number of expected particles for each compartment, N_E(comp), can be calculated by the following equation:

$$N_E(\text{comp}) = N_O(\text{total}) \times (P(\text{comp})/P(\text{total}))$$

where P(comp)/P(total) represents the fraction of observed points falling on the specified compartment.

From the observed and expected distributions, we can calculate the relative deposition index (RDI) which indicates whether the number of observed particles is higher (RDI > 1) or lower (RDI < 1) than the expected number or whether the observed and expected numbers of particles are equal (RDI $= 1$). The RDI is calculated as follows:

$$\text{RDI} = N_O/N_E.$$

The observed and expected distributions can then be subjected to statistical analysis. A convenient way to do this is the chi-squared test.[20] The partial chi-squared (partial χ^2) values are calculated by:

$$\text{partial } \chi^2 = (N_O - N_E)^2/N_E.$$

From the partial chi-squared values, the total chi-squared value is calculated by summing up all partial chi-squared values.

The appropriate null hypothesis to be tested is: "the observed and expected distributions are equal." The total chi-squared value indicates whether or not the null hypothesis can be accepted. If it cannot, a compartment can be regarded as showing preferential particle deposition if two criteria are met: first, the RDI for the compartment is greater than 1 and, second, its partial chi-squared value accounts for a substantial proportion (say, 10% or more) of total chi-squared.

Testing whether the distributions differ between two or more experimental groups

A similar approach to that described above can be used when the observed distributions between two or more groups are to be compared in conjunction with contingency tables.[20] Once again, the observed number of particles (N_O) for each experimental group and compartment can be obtained by direct counting. From the number of observed particles within each group, the number of expected particles (N_E) can be calculated for each compartment, that is, the number of particles that would be expected if the distributions between the groups are not different. The number of expected particles is calculated by contingency table analysis as follows:

$$N_E = (\text{column total} \times \text{row total})/\text{grand total}.$$

Here, the term column refers to an experimental group of cells and the term row to cell compartments. From the numbers of observed and expected particles, the partial chi-squared values are calculated for each compartment and group. Similarly, the total chi-squared value is obtained by adding up all partial chi-squared values.

The corresponding null hypothesis in this case would be: "the observed distributions in different groups are equal". Again, the total chi-squared value indicates whether the null hypothesis has to be rejected. If so, the partial chi-squared values can be used to identify those

groups and compartments that contribute substantially to the overall intergroup difference, that is, the total chi-squared value. Again, a convenient cutoff value for a substantial contribution would be 10% or more of total chi-squared.

Generation of data sets

For the following examples, we can think of a culture of cells known to have the property of phagocytosis as, for instance, alveolar macrophages. The intracellular localization of particles is investigated with respect to the following compartments: cytosol, nucleus, mitochondria, RER/SER, Golgi complex, phagosomes, lysosomes/endosomes. For all other organelles and those structures that cannot be identified unambiguously, a portmanteau compartment named "residual" is created. Data sets on particle number and compartment size were generated for the following three hypothetical scenarios.

Example 1 (Fig. 2). Imagine that a certain type of NP has the following properties: (1) it is not taken up by cells via an active endocytotic pathway, and (2) it is able to cross biological membranes without restriction, which has been shown for some NP.[6] In such a case, it is likely that the particles exposed to a biological system will sooner or later be distributed evenly throughout the entire system. The particles would be able to move freely through the different compartments of the cells.

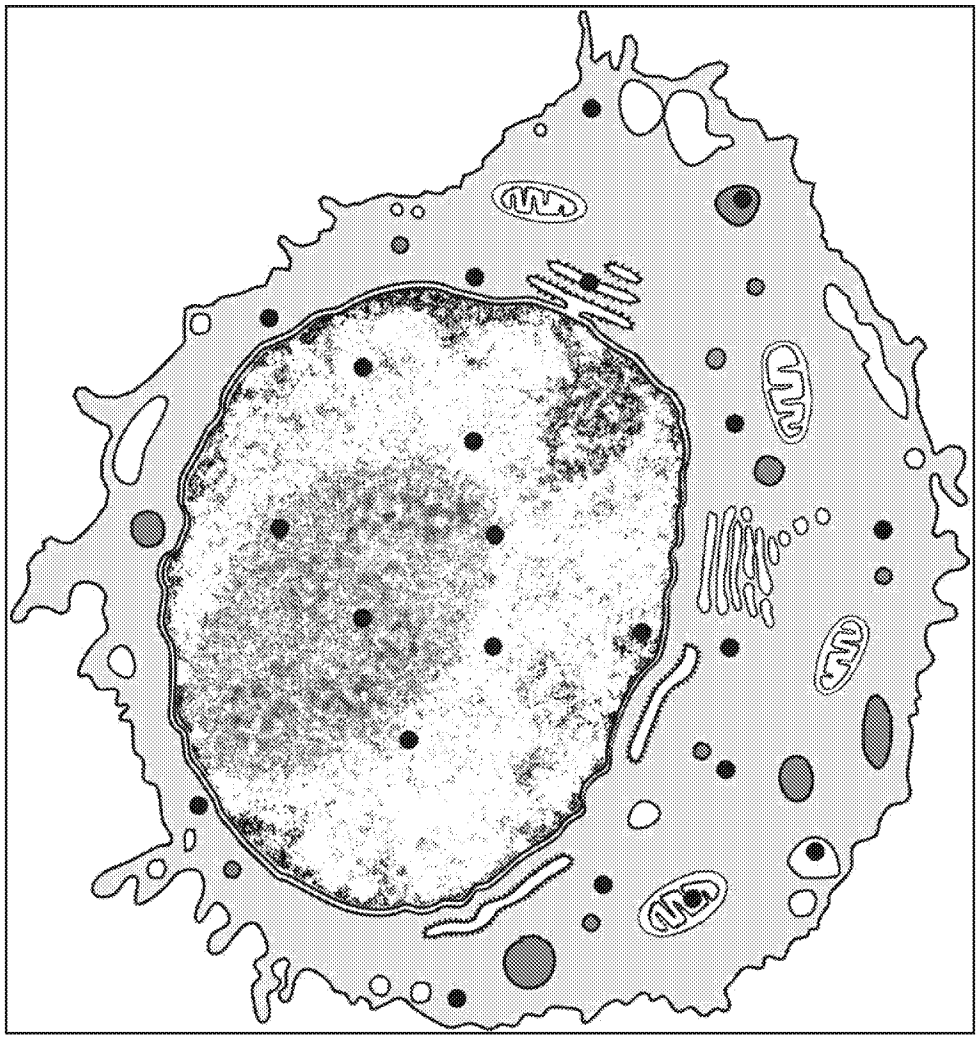

FIG. 2. Random particle distribution (see Example 1). The observed number of particles with each defined compartment is determined by counting and compared with the expected number of particles, which is provided by counting test points as shown in Figure 1. In this case, the particles are randomly distributed within the cell and the observed number of particles with each compartment is similar to the expected number of particles.

As an example, consider a cell culture that is exposed to an aerosol with particles as described above. After fixation and embedding, ultrathin sections are generated and investigated by TEM. In a first step, the volume fractions of different cellular compartments are estimated by test point counting. In a second step, the number of particles associated with each compartment is counted.

In order to simulate this scenario we used test point data based on a realistic compartmentalization of a phagocytosing cell.[14] A randomly chosen number of 587 NP was distributed among the compartments in relation to the number of points for each compartment. The statistical null hypothesis (H0) tested is that there is no significant difference between the observed (NP) and expected (test point) distributions, that is, NP distributions are essentially the same as random.

Example 2 (Fig. 3). Now imagine that the same type of cell was exposed to particles that have different properties of intracellular trafficking: (1) they are taken up by the cells exclusively by an active endocytic mechanism; (2) the endocytic vacuoles (e.g., phagosomes) fuse with endosomes or lysosomes; and (3) for some unknown reason, particles are able to destroy the lysosomal membranes and pass into the cytosol. Preparation and

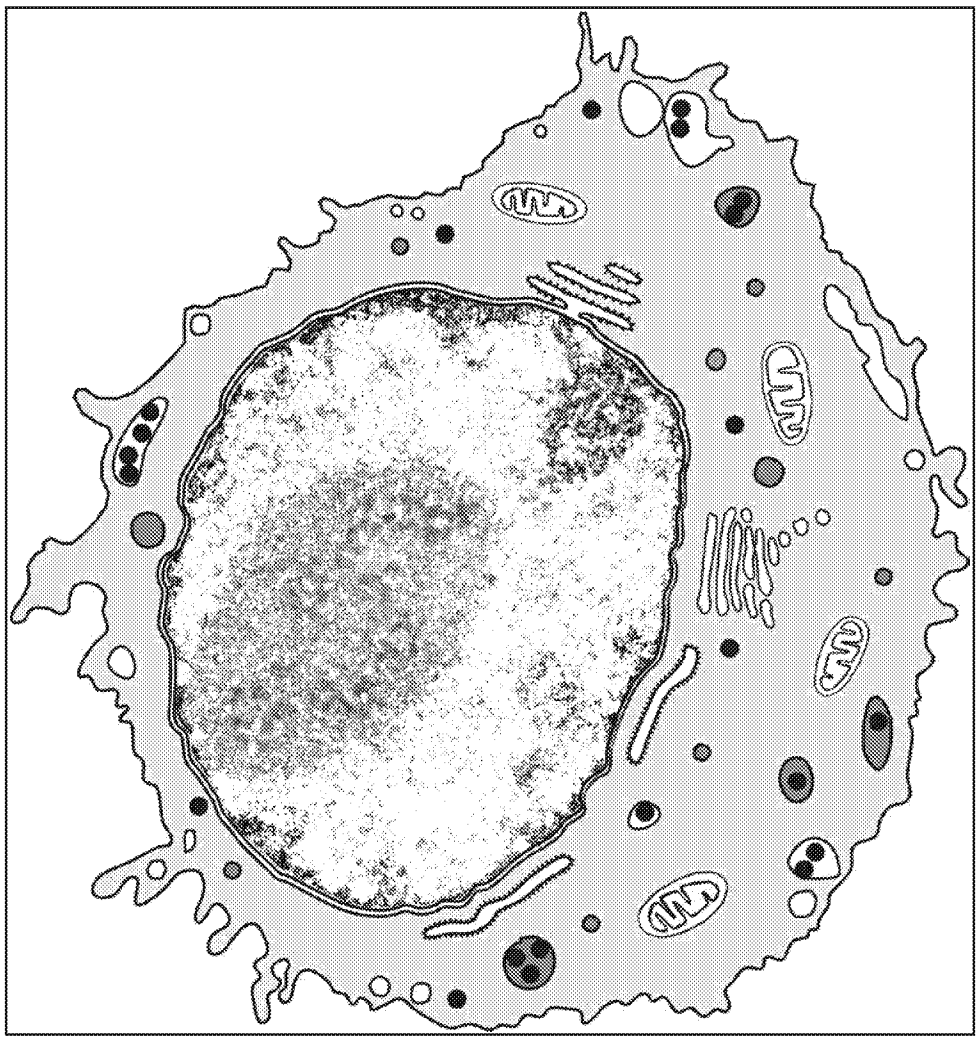

FIG. 3. Nonrandom particle distribution (see Example 2). The observed number of particles with each defined compartment is determined by counting and compared with the expected number of particles which is provided by counting test points as shown in Figure 1. In this case, particles are predominantly located within phagosomes and endosomes/lysosomes. A few particles are also found in the cytoplasm. The observed number of particles in phagosomes and endosomes/lysosomes is higher than it would be expected from their size, which results in a relative deposition index>1. The observed number of particles for the cytoplasm is smaller than expected from compartment size, leading to a relative deposition index<1.

quantification are performed the same way as in Example 1.

This scenario was simulated by using the same set of test point data as in Example 1. A randomly chosen number of 174 NP was distributed among the compartments according to the postulated particle characteristics mentioned above. In this scenario, due to the defined intracellular particle trafficking, there are no particles found in the mitochondria, nucleus, endoplasmic reticulum, or Golgi apparatus. High particle counts are found for the small compartments of phagosomes and lysosomes/endosomes, and some other particles are found in the cytosol of the cells. Assuming that there were a few problems in clearly identifying some of the compartments, there are also other particles in the compartment labelled "residual."

As for Example 1, the appropriate null hypothesis is that there is no significant difference between the observed (NP) and expected (test point) distributions.

Example 3. For this example, imagine that we had exposed the cells to aerosols that contain particles of the same size at different concentrations: aerosol 1 contains only a small concentration of particles, whereas aerosol 2 contains a large concentration. Interestingly, a higher total number of particles, is counted for aerosol 2, which leads to higher particle counts for the individual compartments. The rationale for the contingency table analysis presented in Table 3 is that it allows us to test whether the intracellular distributions at different concentrations of same-sized particles are the same. Without a quantitative approach it is not possible to judge whether the distributions of particles differ between the two different particle concentrations.

In this scenario, the null hypothesis to be tested is that there is no significant difference between the observed NP distributions for aerosols 1 and 2.

RESULTS

Example 1: random particle distribution

From the synthetic data on particle number and point counts the number of expected particles, relative deposition index, and chi-squared values were calculated (Table 1). For the data shown in Table 1, the chi-squared test to compare the observed and expected particle distribution gives a total chi-squared value of roughly 0.63. With 7 degrees of freedom (8-1 compartments by 2-1 columns), a total chi-squared value of 14.07 would be needed to reject the null hypothesis of random distribution at a probability level of $p < 0.05$. Thus, we must accept the null hypothesis that the particles are randomly distributed within the cells.

Example 2: nonrandom particle distribution

Table 2 shows the synthetic data on particle number and point counts as well as the number of expected particles, the RDI, and the chi-squared values in absolute and relative terms for this example. There are three compartments with an RDI > 1 (suggesting that they contain more particles than could be expected from their size)

TABLE 1. SYNTHETIC DATA SET FOR EXAMPLE 1 IN WHICH NANOPARTICLES ENTER CELLS BY AN ENDOCYTOSIS-INDEPENDENT MECHANISM AND BECOME RANDOMLY DISTRIBUTED BETWEEN DIFFERENT INTRACELLULAR COMPARTMENTS

Compartment	*Number of observed particles,* N_O	*Number of observed points,* P	*Number of expected particles,* N_E	*Relative deposition index,* RDI	*Chi-squared values*
Cytosol	310	275	310.43	1.00	0.00
Nucleus	122	110	124.17	0.98	0.04
Mitochondria	59	50	56.44	1.05	0.12
RER/SER	27	25	28.22	0.96	0.05
Golgi complex	12	10	11.29	1.06	0.04
Phagosomes	32	30	33.87	0.94	0.10
Lysosomes/endosomes	19	15	16.93	1.12	0.25
Residual	6	5	5.64	1.06	0.02
Total	587	520	587	1.00	0.63

With 7 degrees of freedom, the total chi-squared value of 0.63 indicates that the null hypothesis (no difference between observed and expected distributions) must be accepted. Essentially, the particles are distributed randomly between cell compartments.

TABLE 2. SYNTHETIC DATA SET FOR EXAMPLE 2, IN WHICH NANOPARTICLES ENTER CELLS BY AN ENDOCYTOSIS-DEPENDENT MECHANISM AND BECOME DISTRIBUTED MAINLY IN COMPARTMENTS OF THE ENDOCYTIC PATHWAY

Compartment	*Number of observed particles,* N_O	*Number of observed points,* P	*Expected particles,* N_E	*Relative deposition index,* RDI	*Chi-squared values*	*Chi-squared values as %*
Cytosol	31	275	92.02	0.34	40.46	3.7
Nucleus	0	110	36.81	0.00	36.81	3.4
Mitochondria	0	50	16.73	0.00	16.73	1.5
RER/SER	0	25	8.37	0.00	8.37	0.8
Golgi complex	0	10	3.35	0.00	3.35	0.3
Phagosomes	94	30	10.04	9.36	702.25	64.0
Lysosomes/endosomes	42	15	5.02	8.37	272.47	24.8
Residual	7	5	1.67	4.18	16.96	1.5
Total	174	520	174	1.00	1097.39	100

With 7 degrees of freedom, the total chi-squared value of 1097.39 indicates that the null hypothesis must be rejected ($p < 0.001$). Only two compartments have partial chi-squares that contribute more than 10% to the total and have RDI values >1. These are the phagosomes and the lysosomes/endosomes.

and five compartments with an RDI < 1 (suggesting that they contain fewer particles than would be expected from their size). The chi-squared test to compare the observed and expected distributions gives a total chi-squared of 1097 which means that the null hypothesis of random particle distribution has to be rejected ($p < 0.001$). Those compartments that contribute substantially to the total chi-squared (a convenient value is 10% or more of total chi-squared) serve to identify the preferential targets of the particles as the phagosomes and lysosomes/endosomes.

Obviously, the number of particles in the cytosol is smaller than would be expected if the distribution was random (RDI < 1). Also, the partial chi-squared value for cytosol does not contribute significantly to the total chi-squared value. It seems that we have identified the preferential targets of the particles but those particles in the cytosol (which, of course, might be of biological interest) cannot be interpreted in a sensible way from this analysis only. It can only be stated that the cytosol is not the preferential target of the particles but that there must have been some process by means of which particles translocated from the phagosomes or lysosomes/endosomes to the cytosol.

Example 3: intergroup comparison of distributions

Table 3 gives the synthetic data set for the intergroup comparison of distributions. The ob-

TABLE 3. SYNTHETIC DATA SET FOR EXAMPLE 3, IN WHICH NANOPARTICLES ARE DELIVERED TO CELLS IN LOW (AEROSOL 1) AND HIGH (AEROSOL 2) CONCENTRATIONS AND THE RESULTING INTRACELLULAR DISTRIBUTIONS ARE COMPARED BY CONTINGENCY TABLE ANALYSIS

Compartment	*Number of observed particles with Aerosol 1*	*Number of observed particles with Aerosol 2*	*Row totals*	*Number of expected particles with Aerosol 1*	*Number of expected particles with Aerosol 2*	*Chi-squared values*	*Chi-squared values as %*
Cytosol	5	36	41	16.16	24.84	7.71, 5.01	17.8, 11.6
Nucleus	1	19	20	7.88	12.12	6.01, 3.91	13.9, 9.0
Mitochondria	1	15	16	6.31	9.69	4.46, 2.90	10.3, 6.7
RER/SER	2	5	7	2.76	4.24	0.21, 0.14	0.5, 0.3
Golgi complex	1	6	7	2.76	4.24	1.12, 0.73	2.6, 1.7
Phagosomes	78	80	158	62.27	95.73	3.97, 2.59	9.2, 6.0
Lysosomes/endosomes	57	60	117	46.11	70.89	2.57, 1.67	5.9, 3.9
Residual	2	5	7	2.76	4.24	0.21, 0.14	0.5, 0.3
Total	147	226	373	147	226	43.35	100

With 7 degrees of freedom, the total chi-squared value of 43.35 indicates that the null hypothesis (no difference between the two group distributions) must be rejected ($p < 0.001$). With Aerosol 1, there are fewer than expected particles on cytosol, nucleus and mitochondria. Aerosol 2 produces more than expected particles on the cytosol.

served and expected particle distributions are compared with the aid of the chi-squared test. With 7 degrees of freedom (8-1 compartments by 2-1 groups) and a total chi-squared of approximately 43.35, $p < 0.001$ and the null hypothesis of identical distribution has to be rejected. Again, the partial chi-squared values indicate those compartments that contribute substantially to the intergroup difference. In this example, the numbers of particles associated with cytosol, nucleus, and mitochondria are smaller than expected with aerosol 1 and higher than expected in the cytosol after exposure to aerosol 2. We conclude that the intracellular particle distributions depend on the concentration of the applied aerosol, and that, at low concentrations, only small numbers are found in the cytosol, nucleus, and mitochondria. At higher particle concentrations, particle number tends to increase in these compartments but only attains significance for one of them, the cytosol.

DISCUSSION

Current knowledge about the interactions of NP with biological systems is rather limited, and there is great consensus that standardized methods are needed to assess the potential health risks of NP.[3,4] Although great efforts have been made to optimize the exposure and develop model systems, little attention has been paid to a quantitative analysis of the intracellular distribution of particles. However, such analysis has the potential to provide hard information about particle translocation characteristics and, if combined with biochemical tools, to identify the correlation between cellular responses and preferential intracellular targets. In order to close this gap, the present study was carried out and provides a description and practical illustrations of novel approaches to analyze the compartment localization of UFP and NP by expressing their spatial distributions as indices of deposition: RDI.

The examples show that point counting and particle counting offer a simple and efficient way of identifying preferential localization of particles if certain preconditions are met. One of the major advantages of the RDI approach is that neither the precise magnification nor the test grid constant (the area associated with each test point) need to be determined. It suffices merely to keep them constant for all fields generated from a given sample of cells or tissue. It needs to be mentioned that the RDI as an estimator of distribution has an equivalent in electron microscopic autoradiography (the index of relative specific activity,[21] and, most recently, in immunoelectron microscopy (relative labelling index).[14] Here, the RDI has been developed in accordance with the relative labeling index[14] but there are certain features that distinguish the use in immunocytochemistry from the use in particle research. Although we recommend this approach for all future studies where NP localization (be it airborne or used for drug delivery) is investigated by means of TEM there are several important factors that may influence the validity and practicability of the RDI as an estimator of particle distribution.

In TEM, the material being subjected to qualitative or quantitative analysis consists of images generated by ultrathin sectioning. Consequently, only an infinitesimally small part of the biological system (organism, organ, tissue, cell) will actually be investigated. This raises the question as to whether the examined sections really represent the biological system. One of the aims of sampling material for microscopy is to give every part of the whole system an equal chance of being included in the analysis. One of the most efficient ways of achieving this is "systematic uniform random sampling" (SURS).[22,23] With SURS, the first item in the sample is selected randomly but then a predetermined interval determines the positions of other items. However, it is important to note that SURS does not end after the sampling of tissue blocks for embedding but continues over the entire (multistage) sampling scheme, that is, the selection of blocks, of sections and, finally, test fields. A convenient way to generate SURS test fields for light microscopy is to start at some random position outside the section and subsequently pass across the whole section using a defined step length in the x and y direction using the stage micrometer controls. For TEM, one consistent corner of the windows between the bars of copper support grids can be used to obtain SURS fields. Once a test field is obtained by SURS it has to enter the analysis. It is not admissible to skip or move test fields simply because other fields look more "representative" or "interesting."

For the purpose of counting particles and test points hitting organelle compartments it is sufficient to randomize location, and it is not neces-

sary to randomize the orientation of the material.[14] However, if particles are suspected to bind to membrane compartments, then section orientation will have to be randomized as well. Although the investigation of particles in the spatial compartments of an organ like the lung can be undertaken without knowledge of orientation, in a cell culture we sometimes want to know where the apical and basal parts of the cells are, especially if sophisticated 3D cell culture models are applied.[7] Consequently, where section orientation is an important sampling consideration, we would propose that, for organs, "isotropic uniform random" (IUR) sections are obtained by the isector[24] or the orientator,[25] whereas, in cell culture models, "vertical uniform random" (VUR) sections should be obtained and combined with cycloid test systems.[26] As the present paper predominantly deals with organelle compartments (for which section orientation is not critical), the latter methods are not explained further.

Currently, knowledge about the entry and subcellular distribution and trafficking of NP is rather limited and, basically, when a new experimental system is being investigated, all organelle compartments of a cell must be suspected to contain particles. For example, particles that follow a strict endocytotic pathway will be located in the corresponding membrane-bound organelles, whereas particles that are able to enter and move freely within the cells may be observed in every organelle compartment. In immunogold labeling, a restriction of subcellular compartments to 10–12 is proposed,[14,15] and this offers a reasonable frame for the present purposes as well. A first approach would be to distinguish between, say, the nucleus, mitochondria, endoplasmic reticulum, Golgi complex, phagosomes, endosomes, lysosomes, cytosol, and residual cellular structures. For practical reasons, it needs to be mentioned that the number of compartments chosen also depends on the quality of the tissue preparation and may be limited by the difficulty or impracticability of unambiguously distinguishing between several compartments. However, the number of compartments used in a study should still be enough to allow a meaningful biological interpretation. If trafficking through the cell is to be determined in the context of a time series of exposure, one might also be interested in splitting compartments (e.g., early and late endosomes) that cannot be distinguished solely on the basis of their morphology. In such cases, immunogold labeling of compartments with specific antigens may be useful in aiding identification.

Obviously, it is a prerequisite that the NP can be recognized unambiguously. First, this implies that the magnification level used for counting particles must be sufficient to resolve particles even though they may be only a few nanometers in size. Working at magnifications too low to resolve smaller particles may result in a size-dependent bias if, say, larger NP show different compartment distributions. Second, in reality, particles are distributed through the entire thickness of the section (40–60 nm). Therefore, very small particles may be overlooked if they are not near the cut surface of the section or lack sufficient contrast themselves or are lost because their presence is masked by a surrounding electron-dense matrix or neighboring electron-dense structures such as mitochondria. Given the basic principles of stereology, the number of particles cannot be estimated from one thin section,[11,23,26] but instead, would require the use of the disector.[27] However, in the case of NP, the physical disector (two "thin" sections a certain distance apart) has limited utility and use of the optical disector (one "thick" section through which a series of focal planes is traversed) would require the application of electron tomography, which is not routinely available yet.

For reasons of practicability and efficiency, the ultrathin sections are treated as a two-dimensional system and NP are regarded as if in the same plane. Although this introduces a potential bias, because larger sized particles have higher chances of being sectioned than smaller ones, the error may be minimized by keeping sections as thin as possible and by adopting NP that are relatively homogeneous in size. A third point arises from the fact that cellular structures (such as ribosomes, mitochondrial, or glycogen granules) or artefacts (like particulate precipitations of contrasting media) may mimic NP with similar electron density and shape. In these cases, the analysis needs to be confirmed by element analysis using energy filtering transmission electron microscopy.[5,28] With particles that are electron lucent (e.g., polystyrene particles), this will be much more difficult. In those cases, it needs to be checked whether they can be visualized by immunogold labeling, which allows the counting of gold particles at the section surface as an indirect NP count.

Due to the distribution of particles through the entire section thickness there may be practical difficulty in assigning particles to their appropriate compartment. For comparing different tissue compartments this may not be problematic. At the cellular level, however, we may have to define smaller compartments which—if they are at the nanoscale—will also be distributed throughout the section thickness and, in consequence, difficult or impossible to identify. This may be made worse if there is poor preservation of ultrastructure due to technical procedures, for example cryosubstitution for immunogold labeling. In such circumstances, a sensible strategy is to include one compartment with the label "don't know," that embraces all structures that cannot be identified.

The quantitative method presented here incorporates the statistical analysis of the observed distribution of NP within tissue or intracellular compartments. It is worth emphasizing that this sort of analysis is not meant to replace the qualitative analysis that should precede any quantitative investigation. Nor can it replace the critical scientific evaluation and interpretation of results. In most cases of particle deposition, the size of the particles varies within a certain range. Because it is well accepted that the size, surface, and shape of particles contribute to different biological reactions,[29] it should be taken into account that exposure of an experimental system to an aerosol may include exposure to particle fractions with different biophysical properties. Counting all particles independent of their size may therefore estimate the distribution of different subgroups of particles that, separated from each other, may have different intracellular distributions. In such case, care has to be taken not to draw the wrong conclusion.

In contrast to immunocytochemistry, where a small number of immunogold particles associated with compartments that obviously do not contain the labeled antigen can be neglected and may represent the labeling noise, the occurrence of NP within a compartment has to be taken seriously. Although other compartments may contain a greater number of NP and may appear as preferential targets of the particles, a small number of NP within a non-preferential compartment may also have biological significance (see Example 2).

Apart from these sources of bias, the precision of the estimates must also be considered. The precision of both particle and test point distributions is dependent on the number of particles and points counted. Stereological analysis based on test point data have demonstrated that 100–200 points falling on a given compartment or reference space is sufficient to guarantee that the overall observed variance in a study design is governed principally by the biological variation between subjects (or, here, between cell culture replications) and not by the variation due to the precision of measuring and sampling procedures.[30,31] Although increasing the number of compartments that are investigated surely increases the biological resolution of particle localization, it also requires a larger amount of work if particle counts on every compartment are to be analyzed with equal precision.[14] Therefore, while keeping separate compartments with different functional characteristics, it is sensible to give careful consideration as to which compartments are most important to reach a reasonable biological conclusion and adequately test a relevant null hypothesis. If, for example, the localization of particles is to be correlated with markers of oxidative stress or genotoxicity, it may be less important to distinguish between early and late endosomes. By the same token, if the emphasis is placed on the endocytotic pathway, the differentiation of vesicular structures may be rather more pertinent than distinguishing between nuclei and mitochondria.

An additional factor influencing the decision regarding the number of compartments relates to the statistical testing. For the chi-squared analysis to be effective, it is preferable that no expected number of particles should be less than 1, and no more than 20% of them should be less than 5 [see[15]]. If these requirements cannot be satisfied, it will be adviseable to count more particles or, where this is not feasible, to reduce the number of compartments (either by omitting them or by combining them into a larger portmanteau compartment). For intergroup comparisons of particle distributions, analyses are reasonably insensitive to group differences in the total numbers of particles per cell provided that these are not excessive.[15]

In conclusion, the present study has illustrated that, when used in conjunction with randomized sampling protocols and chi-squared analyses, simple counts of UFP and NP and test points can provide an inexpensive and efficient way to compare distributions within and between speci-

mens. The basic principles are applicable at several levels of biological organization from the whole organism down to the subcellular level.

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